Final Scientific Report

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Project Title: IQD1 Function in Defense Responses

Investigators	**Institutions**
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Collaborating Investigators: Reddy Anireddy	**Colorado St. University**

Keywords; Glucosinolates; Calmodulin; Calcium signaling;

Abbreviations commonly used in the report, in alphabetical order**:**

AD= activation domain
DB= DNA binding domain
DEX= dexamethasone
CaM = calmodulin
ET = ethylene
GR= glucocorticoid receptor
IQD1 = IQ domain 1
$IQD1^{OXP}$ =IQD1 overexpression line
JA = jasmonic acid
NPR1 = non-expressor of PR1 gene
SA = salicylic acid
TAP= tandem affinity purification
Y2H= yeast two hybrid

Budget: IS: $130,000 US: $ 200,000 Total: $ 330,000

Maggie _____ _____ _____

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	1		3	
Submitted, in review, in preparation	1			
Invited review papers	1			
Book chapters				
Books				
Master theses				
Ph.D. theses				
Abstracts				
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	-	-	Skype conferences	3
Longer Visits (Sabbaticals)	-	-	-	

Description Cooperation:

The scientific collaboration between the Israeli and American groups is primarily synergistic. The Israeli group (Levy's lab) were responsible for identifying transcriptional targets (RNAseq), and biological activity of IQD1 forms (HPLC analysis, defense responses and hormone signaling). As such they did RNAseq analysis of IQD^{OXP} as

compare to WT. The American groups were responsible for protein-protein and protein-nucleic acid interactions (Reddy's lab; yeast two-hybrid screen, TAP-tag and ChIP-Seq analyses) and for the IQ67 domain mapping (Zielinski's lab: mutations and CaM-binding assays). All groups will jointly verify and characterize IQD1 targets later on (qRT-PCR, BiFC). American groups received the IQD1 cDNA from Levy's group and they used it to construct the GAL4, BiFC, TAP-tag and the IQ67 mutants. Reddy's group also provided with Arabidopsis IDQ1 knockout seeds from Levy's group and they used it to construct the TAP-tagged transgenic lines. Levy's group analyzed the TAP tagged lines from Reddy's lab for GS content and did defense response analysis on this line. AtSR1 (an IQD1 interactor) loss of function and complementation lines were provided By the Reddy's lab to Levy's group and Levy's group analyzed those line for GS content and *B. cinerea* resistance assays. Levy's group provided Zielinski's lab seeds of IQD1 knockout lines for transformation with the IQD67 mutant constructs. The major results of the project were necessarily stem from the symbiosis of the combined experimental approaches, and eventually lead to a joint publication in a leading pear reviewed journal and for an invited review which is currently under preparation.

Patent Summary(numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	-	-	-	-
Issued (allowed)				
Licensed				

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Abstract

The main objective of the proposed research was to study IQD1's mechanism of action and elucidate its role in plant protection. Preliminary experiments suggest that IQD1 binds CaM in a Ca^{2+}-dependent manner and functions in general defense responses. We propose to identify proteins and genes that interact with IQD1, which may provide some clues to its mechanism of action. We also plan to dissect IQD1's integration in defense pathways and to study and modulate its binding affinity to CaM in order to enhance crop resistance. Our specific objectives were: (1) Analysis of IQD1's CaM-binding properties; (2) Identification of IQD1 targets;(3) Dissection of IQD1 integration into defense signaling pathways. Analysis of IQD1's CaM-binding properties defined four potential classes of sequences that should affect CaM binding: one is predicted to raise the affinity for Ca^{2+}-dependent interaction but have no effect on Ca^{2+}-independent binding; a second is predicted to act like the first mutation but eliminate Ca^{2+}-independent binding; a third has no predicted effect on Ca^{2+}-dependent binding but eliminates Ca^{2+}-independent binding; and the fourth is predicted to eliminate or greatly reduce both Ca^{2+}-dependent and Ca^{2+}-independent binding. Following yeast two hybrid analysis we found that IQD1 interact with AtSR1 (*Arabidopsis thalianaSIGNAL RESPONSIVE1*), a calcium/calmodulin-binding transcription factor, which has been shown to play an important role in biotic and abiotic stresses. We tested IQD1 interaction with both N-terminal or C-terminal half of SR1. These studies have uncovered that only the N-terminal half of the SR1 interacts with the IQD1. Since IQD1 has an important role in herbivory, its interaction with SR1 suggests that it might also be involved in plant responses to insect herbivory. Since AtSR1, like IQD1, is a calmodulin-binding protein and the mutant showed increased sensitivity to a herbivore, we analyzed WT, *Atsr1* and the complemented line for the levels of GS to determine if the increased susceptibility of *Atsr1* plants to *T. ni* feeding is associated with altered GS content. In general, *Atsr1* showed a significant reduction in both aliphatic and aromatic GS levels as compared to WT. In order to study IQD1's molecular basis integration into hormone-signaling pathways we tested the epistatic relationships between IQD1 and hormone-signaling mutants. For that purpose we construct double mutants between $IQD1^{OXP}$ and mutants defective in plant-hormone signaling and GS accumulation. Epitasis with SA mutant *NahG* and *npr1-1* and JA mutant *jar1-1* suggested *IQD1* function is dependent on both JA and SA as indicated by *B. cinerea* infection assays. We also verified the glucosinolate content in the crosses siblings and found that aliphatic GSL content is reduced in the double transgenic plants NahG:$IQD1^{OXP}$ as compare to parental lines while the aliphatic GSL content in the *npr1-1:*$IQD1^{OXP}$ and jar1-1: $IQD1^{OXP}$ double mutants was intimidated to the parental lines. This suggests that GSL content dependency on SA is downstream to IQD1. As a whole, this project should contribute to the development of new defense strategies that will improve crop protection and reduce yield losses and the amount of pesticides required; these will genuinely benefit farmers, consumers and the environment.

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Achievements:

1. **Analysis of IQD1's CaM-binding properties**

We analyzed the coding sequence of IQD1 using the algorithms of Yap et al. (http://calcium.uhnres.utoronto.ca/ctdb/pub_pages/search/index.htm) to predicted the effects of point mutations on the likelihood of CaM-binding to the region between amino acids 94 and 124. This region defines both Ca^{2+}-dependent and a Ca^{2+}-independent binding sites for CaM. These analyses defined four potential classes of sequences that should affect CaM binding: one (E108Q) is predicted to raise the affinity for Ca^{2+}-dependent interaction but have no effect on Ca^{2+}-independent binding; a second (E108Q, I114A) is predicted to act like the first mutation but eliminate Ca^{2+}-independent binding; a third (I114A) has no predicted effect on Ca^{2+}-dependent binding but eliminates Ca^{2+}-independent binding; and the fourth (F101G, I112G, I114A) is predicted to eliminate or greatly reduce both Ca^{2+}-dependent and Ca^{2+}-independent binding. These mutations are currently under construction.

2. **Identification of IQD1 targets**

Genome-wide transcriptional profiling analysis. Since the induction system with DEX could not be calibrated with our selected 35S::IQD1-GR lines we are currently analyzing our data from RNA sequencing method (RNAseq) of the available constitutive *$IQD1^{OXP}$* line as compare to WT with and without *B. cinerea* infection.

IQD1 interacts with SR1/CaMTA3, a calmodulin-binding transcription factor. Following yeast two hybrid analysis we found that IQD1 interact with AtSR1 (*Arabidopsis thalianaSIGNAL RESPONSIVE1,* also called CaMTA3), a calcium/calmodulin-binding transcription factor, which has been shown to play an important role in biotic and abiotic stresses. We tested IQD1 interaction with both N-terminal or C-terminal half of SR1. These studies have uncovered that only the N-terminal half of the SR1 interacts with the IQD1.

Atsr1 mutant shows increased susceptibility to herbivory. The observed interaction of AtSR1 with the IQD1 (described above) and its general role in biotic and abiotic stresses indicated a potential role for AtSR1 in herbivory. In an effort to define the role of *AtSR1* in insect herbivory, we assayed its function in plant resistance to herbivory. Four days post-incubation with larvae of the generalist herbivore *Trichoplusia ni*, *Atsr1* plants had become severely defoliated, supporting

significantly higher larval weight gains as compared to those of wild-type (WT) fed insects. Similarly, in detached leaf assays loss of *AtSR1* function resulted in larval weight gains nearly 3 times greater than that of wild-type fed insects only 12 hours after inoculation. Constitutive expression of *AtSR1* in *Atsr1* plants (*AtSR1;Atsr1*) restored wild-type levels of resistance further indicating the increased susceptibility of the mutant is a direct result of loss of *AtSR1* function.

AtSR1, like IQD1, is a positive regulator of glucosinolate biosynthesis in Arabidopsis. *IQD1* encodes a calmodulin-binding protein involved in the positive regulation of global GS levels during biotic stress responses. Since AtSR1, like IQD1, is a calmodulin-binding protein and the mutant showed increased sensitivity to a herbivore, we analyzed WT, *Atsr1* and the complemented line for the levels of GS to determine if the increased susceptibility of *Atsr1* plants to *T. ni* feeding is associated with altered GS content. In general, *Atsr1* showed a significant reduction in both aliphatic and aromatic GS levels as compared to WT. Levels of 3-methylsulfinylpropyl (3MSOP), 8-methylsulfinyloctyl (8MSOO), benzyl and, to a greater degree, indol-3-ylmethyl (I3M) and 4-methylsulfinylbutyl (4MSOB) were all significantly decreased in the mutant. In the complemented plant (*AtSR1;Atsr1)* GS levels were restored to WT level, suggesting that the reduced level of GS in the mutant is due to loss of *AtSR1* function.

Expression of genes associated with glucosinolate biosynthesis are significantly altered in the *Atsr1* mutant. Previously, transcriptome analysis identified *WRKY53* expression to be significantly up-regulated in the *Atsr1* mutant. *WRKY53* encodes a calmodulin-binding transcription factor that functions in the regulation of GS hydrolysis. WRKY53 interacts with ETHIOSPECIFIER PROTEIN (ESP) and the two antagonistically regulate the conversion of GS to nitriles and isothiocyanates. In an effort to further define the role of *AtSR1* in GS metabolism, we assayed the mutant for altered expression of *ESP* and other genes related to indole GS biosynthesis and hydrolysis. Basal expression of *WRKY53* and *WOUND RESPONSIVE3* (*WR3*), encoding a high-affinity nitrate transporter, was significantly higher in *Atsr1* plants as compared to wild-type (Laluk et al., 2012). Surprisingly, wounding led to decreased *WRKY53* and *WR3* transcript accumulation in the mutant, which is in direct contrast to their WT pattern of expression (Laluk et al., 2012). Additionally, basal and wound-induced levels of both genes were dramatically reduced in *AtSR1;Atsr1* plants suggesting *AtSR1* functions as a negative regulator of *WRKY53* and *WR3* expression. Expression of *ESP* was also altered in *AtSR1;Atsr1*, showing a significant reduction in response to wounding, yet levels were not affected by loss of *AtSR1* function.

Tandem affinity purification of IQD1 protein complexes. To isolate IQD1 interacting proteins *in vivo* we cloned IQD1 into a TAP (tandem affinity purification). To test if the fusion protein is functional and that there is no competition with the endogenous protein we transformed *iqd1* mutant (ABB8). The mutant phenotype of *iqd1*was complemented in this line, suggesting that the fusion protein is functional. The complemented line is being used for tandem affinity purification and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis.

Analysis of interaction of IQD1 and SR1 using bimolecular fluorescence complementation (BiFC) assay. To verify in vivo interaction of SR1 with IQD1 *in planta* we constructed and verified several bimolecular fluorescence complementation (BiFC) constructs. Interaction of IQD1 with SR1 is beingtested this week by transiently expressing these constructs in protoplasts. We are also currently testing transiently co-expression of SR1-YFP and IQD1-GFP constructs in Arabidopsis roots.

3. Dissection of IQD1 integration into defense signaling pathways

Epistasis analysis between IQD1 and hormone-signaling components

In order to study IQD1's molecular basis integration into hormone-signaling pathways we tested the epistatic relationships between IQD1 and hormone-signaling mutants. For that purpose we construct double mutants between $IQD1^{OXP}$ and mutants defective in plant-hormone signaling and GS accumulation. Epitasis with SA mutant *NahG* and *npr1-1* and JA mutant *jar1-1* suggested *IQD1* function is dependent on both JA and SA as indicated by *B. cinerea* infection assays. We also verified the glucosinolate content in the crosses siblings and found that aliphatic GSL content is reduced in the double transgenic plants NahG:$IQD1^{OXP}$ as compare to parental lines while the aliphatic GSL content in the *npr1-1:*$IQD1^{OXP}$ and jar1-1: $IQD1^{OXP}$ double mutants was intimidated to the parental lines. This suggests that GSL content dependency on SA is downstream to IQD1.

Publications:

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